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OUTI LYYTINEN

MOLECULAR DETAILS OF THE DOUBLE-STRANDED RNA VIRUS REPLICATION AND ASSEMBLY

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Molecular details of the double-stranded RNA virus replication and assembly

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University of Helsinki

ACADEMIC DISSERTATION

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"How lucky I am to have something that makes saying goodbye so hard"

Winnie the Pooh. A. A. Milne

LIST OF THE ORIGINAL PUBLICATIONS

The Thesis of is based on the following publications, which are referred to in the text by their Roman numerals:

I. Collier AM*, Lyytinen OL*, Guo YR, Toh Y, Poranen MM, Tao YJ. 2016. Initiation of RNA polymerization and polymerase encapsidation by a small dsRNA virus. PLOS Pathogens, 12; e1005523. *These authors contributed equally.

II. Lyytinen OL, Starkova D, Poranen MM. 2019. Microbial production of lipid-protein vesicles using enveloped bacteriophage phi6. Microbial Cell Factories, 18; 29.

The doctoral candidate's contribution to the articles included in this Thesis:

I. The doctoral candidate OLL was involved in the experimental design, produced the template RNAs for the enzymatic analyses, optimized and performed the biochemical activity assays, and analyzed the data. OLL also prepared the figures and wrote those parts of the manuscript that are related to the enzymatic studies.

II. The doctoral candidate OLL did experimental design, constructed many of the expression strains, produced and purified phi6-specific vesicles, analyzed biochemical and physical properties of the vesicles, visualized the vesicle producing cells using fluorescence and electron microscopy, and purified vesicles using electron microscopy, analyzed the data, prepared the figures and wrote the manuscript.

ABBREVIATIONS

aa	amino acid
bp	base pair
CM	cytoplasmic membrane
CP	capsid protein
D	aspartic acid residue (amino acid)
DNA	deoxyribonucleic acid
dsDNA	double-stranded deoxyribonucleic acid
dsRNA	double-stranded ribonucleic acid
EM	electron microscopy
GFP	green fluorescent protein
GTP	guanosine triphosphate
HCV	hepatitis C virus
hPBV	human picobirnavirus
ICTV	International Committee for the Taxonomy of Viruses
kbp	kilo base pairs
kDa	kiloDalton, 1000 Daltons, 1 Dalton = 1 atomic mass unit
mRNA	messenger ribonucleic acid
(-)ssRNA	negative-sense single-stranded ribonucleic acid

NTPase	nucleotidyl triphosphatase
NTP	nucleoside triphosphate
OM	outer membrane
ORF	open reading frame
PBV	picobirnavirus
PBV2	second genomic segment of human picobirnavirus
PC	polymerase complex
(+)ssRNA	positive-sense single-stranded ribonucleic acid
RdRp	RNA-dependent RNA polymerase
RNA	ribonucleic acid
rPBV	rabbit picobirnavirus
ssRNA	single-stranded ribonucleic acid
T	triangulation number
TNTase	terminal nucleotidyl transferase
UTR	untranslated region
VLP	virus-like particle
wt	wild type
Å	ångström, 0.1 nanometers

ABSTRACT

Viruses are obligate parasites infecting the cells from all the three domains of life: *Bacteria*, *Archaea* and *Eukarya*. Ribonucleic acid (RNA) viruses contain ribonucleic acid as their genomic element instead of deoxyribonucleic acid (DNA). There are three main types of RNA viruses: positive-sense single-stranded [(+)ssRNA], negative-sense single-stranded [(-)ssRNA] and double-stranded RNA (dsRNA) viruses. This Thesis is focused on revealing molecular details of replication and assembly of two dsRNA viruses: Pseudomonas phage phi6 (phi6) and human picobirnavirus (hPBV).

Double-stranded RNA viruses need to carry an RNA-dependent RNA polymerase (RdRp) inside their virion to the host in order to be able to replicate their genome. We characterized the hPBV RdRp enzymatically and structurally and revealed the similarities of this RdRp to the other known small dsRNA virus RdRps like phi6 RdRp, which has been extensively studied. We showed that hPBV RdRp has a canonical cupped right-hand polymerase structure, it can replicate and transcribe homologous and heterologous template RNA in the absence of capsid proteins and it also possesses terminal nucleotidyl transferase activity. This is only the second dsRNA virus RdRp reported with this activity.

The assembly of these two viruses is relatively different due to the differences in their structures. Phi6 has three layers and a lipid-protein envelope as its outermost layer whereas hPBV does not have any lipids in its structure and is composed of only one layer of capsid proteins surrounding the dsRNA genome. The assembly of the inner protein layers of phi6 is very well-known whereas as the envelope formation and the assembly of hPBV capsid layer is largely uncharacterized. Our results suggest that hPBV might use a co-assembly of its capsid proteins and

genomic RNA precursors as its assembly strategy. The envelope assembly of phi6 was studied expressing phi6 membrane proteins in *Escherichia coli* bacteria. Our results revealed that only one small membrane protein P9 can induce phi6-specific vesicle formation in *E. coli* cells. Also, heterologous green fluorescent protein can be added to the vesicles by co-expressing non-structural P12 protein.

This study reveals interesting molecular details about the genome replication and assembly of hPBV, a relatively unknown opportunistic human pathogen, and the envelopment process of phi6. These results are biologically interesting and may have also biotechnological applications in the future.

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1. INTRODUCTION

Viruses are extremely abundant and found everywhere in the world. It has been estimated that there are approximately 10^{31} viruses on Earth (Breitbart and Rohwer, 2005). They are obligate parasites relying totally on the metabolism of their host cells. Viruses can infect members of all the three domains of life: *Bacteria*, *Archaea* and *Eukarya*. In fact, one way to classify viruses is by the host cell they infect. Other methods for classification of viruses include the genetic material of the virus, morphology, i.e. the shape and appearance of the virion, and the structure of the capsid protein (Ravantti et al., 2013). Virion means the viral particle existing outside the host cell. Classifying viruses based on the type of their genetic material and the relationship of the genome to the messenger ribonucleic acid (mRNA) is called the Baltimore classification (Baltimore, 1971). Viruses which have a lipid-protein envelope around their protein capsid are called enveloped viruses and those which lack the envelope, naked viruses. The proteinaceous capsid of the virion can represent e.g. icosahedral or helical symmetry or a complex tail-containing structure. Classification of viruses based on structural lineages has been suggested. This means dividing viruses into different lineages by the structure of their major capsid protein (Bamford et al., 2002; Ravantti et al., 2013).

1.1. RNA viruses

Viruses that possess ribonucleic acid (RNA) as their genomic material are called RNA viruses or riboviruses. There are three main types of RNA viruses: positive-sense single-stranded [(+)ssRNA], negative-sense single-stranded [(-)ssRNA] and double-stranded RNA (dsRNA) viruses. In addition, retroviruses also contain an RNA genome, but they are generally not regarded as RNA viruses since they

need a deoxyribonucleic acid (DNA) intermediate of their genome for replication. A common feature to all RNA viruses is their RNA-dependent RNA polymerase (RdRp) enzyme that is essential for the replication of the genomic material. Viruses with (-)ssRNA and dsRNA genomes have to package their RdRps inside the viral capsids and carry the polymerases to the host cell in order to be able to replicate. Thus, the incorporation of the RdRp during virion assembly is a crucial step of their lifecycles. (+)ssRNA viruses can use genomic RNA directly as mRNAs for their protein translation and thus the packaging of RdRps into their virions is not crucial for their replication. Currently there are 47 different (+)ssRNA virus families recognized by the International Committee for the Taxonomy of Viruses (ICTV) and the amount of different genus and species is even larger (ICTV Master Species List). (+)ssRNA viruses include e.g. norovirus (family *Caliciviridae*), Zika virus (*Flaviviridae*), hepatitis C virus (*Flaviviridae*), poliovirus (*Picornaviridae*) and tobacco mosaic virus (*Virgaviridae*). Since the genome of (-)ssRNA viruses cannot be used directly as mRNA their RdRp starts the viral replication by transcribing the negative-sense genome to positive-sense mRNAs. Currently there are 27 different (-)ssRNA virus families (ICTV Master Species List). Viruses with a (-)ssRNA genome contain many important pathogens such as rabies (*Rhabdoviridae*), measles (*Paramyxoviridae*), Ebola (*Filoviridae*) and influenza viruses (*Orthomyxoviridae*) (Ortín and Martín-Benito, 2015).

1.2. Double-stranded RNA viruses

DsRNA viruses are currently classified into eleven different virus families by the ICTV (ICTV Master Species List) (Table 1). Reoviruses and cystoviruses are relatively complex dsRNA viruses since they both have three concentric virion layers. All the three layers of reoviruses are composed of protein, but cystoviruses

have a lipid-protein envelope as their outermost layer. Proteins in the second and third layer of reoviruses, in the second layer of cystoviruses and in the only protein layer of birnaviruses are arranged on an icosahedral T=13 (triangulation number = 13) lattice. The innermost layer of reo- and cystoviruses and the only capsid forming layer of the small dsRNA viruses (amalga-, chryso-, megabirna-, partiti-, picobirna-, quadri- and totiviruses) follows T=1 icosahedral symmetry (Table 1). All the others, except chryso- and quadriviruses, have a homodimer as an asymmetric unit in their capsids (Table 1) (Luque et al., 2010; Poranen and Bamford, 2012). This unique structural organization, found only from dsRNA viruses, is sometimes referred to as the "T=2" structure (Grimes et al., 1998), although, triangulation number 2 is not theoretically possible (Caspar and Klug, 1962). Interesting exceptions among dsRNA viruses are chrysoviruses which have an authentic T=1 capsid structure in which the capsid proteins (CPs) have two similar domains resulting from a gene duplication (Caston et al., 2003; Caston et al., 2013; Luque et al., 2010) and quadriviruses which have a heterodimer as an asymmetric unit (Luque et al., 2016). The structural similarity and the functional similarities in genome replication indicate a shared common ancestor to these dsRNA viruses (Bamford et al., 2002; Luque et al., 2010). However, there are also dsRNA virus isolates which have totally different capsid structure and life cycle. These include a mycovirus Colletotrichum camelliae filamentous virus 1 which forms filamentous particles (Jia et al., 2017), another mycovirus, Aspergillus fumigatus tetramycovirus-1, of which genomic dsRNA is infectious (Kanhayuwa et al., 2015) and the members of the *Endornaviridae* family, which have not been detected to produce any virus like particles (VLPs) (Fukuhara and Gibbs, 2012). In this Thesis I will focus on two dsRNA virus species *Human picobirnavirus* and *Pseudomonas virus phi6* which are the type species of the *Picobirnaviridae* and *Cystoviridae* families, respectively (Table 1).

Table 1. Double-stranded RNA viruses

Family	Example genera	Type species	Hosts	Genome segments	Structure[1)]
Amalgaviridae	*Amalgavirus*	*Southern tomato virus*	plants	1	
Birnaviridae	*Aquabirnavirus*	*Infectious pancreatic necrosis virus*	fish, molluscs, crustaceans	2	
	Avibirnavirus	*Infectious bursal disease virus*	birds		
Chrysoviridae	*Alphachrysovirus*	*Penicillium chrysogenum virus*	fungi	4	
Cystoviridae	*Cystovirus*	*Pseudomonas virus phi6*	bacteria	3	
Endornaviridae	*Alphaendornavirus*	*Oryza sativa alphaendornavirus*	plants, fungi, oomycetes	1	
Megabirnaviridae	*Megabirnavirus*	*Rosellinia necatrix megabirnavirus 1*	fungi	2	
Partitiviridae	*Alphapartitivirus*	*White clover cryptic virus 1*	plants	2	
	Cryspovirus	*Cryptosporidium parvum virus 1*	protozoa		
Picobirnaviridae	*Picobirnavirus*	*Human picobirnavirus*	mammals, birds, reptiles	2	
Quadriviridae	*Quadrivirus*	*Rosellinia necatrix quadrivirus 1*	fungi	4	
Reoviridae	*Orbivirus*	*Bluetongue virus*	vertebrates, arthropods	10	
	Rotavirus	*Rotavirus A*	vertebrates	11	
Totiviridae	*Totivirus*	*Saccharomyces cerevisiae virus L-A*	fungi, protozoa	1	

[1)] Blue: T=1 protein layer; green: T=13 protein layer; yellow: lipid membrane; purple: dsRNA genome

1.2.1. *Picobirnaviridae*

Viruses in the family *Picobirnaviridae* are small, non-enveloped dsRNA viruses. They have a single layered capsid structure of approximately 35–40 nm in diameter (Duquerroy et al., 2009). The capsid is composed of 120 asymmetric homodimers and thus follows the T=1 or "T=2" icosahedral symmetry (Duquerroy et al., 2009). Picobirnaviruses (PBVs) have two linear dsRNA genome segments. The length of the first and the second segment varies between 2.3–2.6 and 1.5–1.9 kilo base pairs (kbp), respectively (Delmas et al., 2019; Wakuda et al., 2005). PBVs are considered putative opportunistic pathogens associated with diarrhoea and gastroenteritis. *Picobirnaviridae* family has only one genus *Picobirnavirus* which has two ICTV ratified species: *human picobirnavirus* and *rabbit picobirnavirus* (Delmas et al., 2019). In addition, a plethora of different PBV strains infecting also other mammals, birds and reptiles have been reported worldwide (Day et al., 2010; Ganesh et al., 2014; Ludert et al., 1991; Pereira et al., 1988). All the structural data of the picobirnaviruses is from the rabbit picobirnavirus (rPBV) (Duquerroy et al., 2009). The rPBV CP undergoes autocatalytic cleavage during viral assembly and the N-terminal peptide of the CP remains inside the capsid associated with the dsRNA (Duquerroy et al., 2009). Recently, it was proposed, that PBVs would not be viruses of mammals, birds and reptiles, but instead, infect prokaryotes living in the intestines of these animals (Krishnamurthy and Wang, 2018). This hypothesis was based on the discovery of bacterial ribosomal binding sites in all the investigated picobirnaviral genomes (Krishnamurthy and Wang, 2018).

Human picobirnavirus (hPBV) is found frequently in stool samples of children and immunocompromised persons with gastroenteritis (Bhattacharya et al., 2007; Ganesh et al., 2011; Giordano et al., 2008). Genome segment 1 (PBV1) is 2525

base pairs (bp) long and it contains two open reading frames (ORFs) (Fig. 1) (Wakuda et al., 2005). The first ORF encodes a protein of 224 amino acids (aa) with an unknown function and the second ORF the CP of the virus (Fig. 1). The second genome segment (PBV2; 1745 bp) encodes an RdRp which is responsible for the replication and transcription of the viral genome (Fig.1).

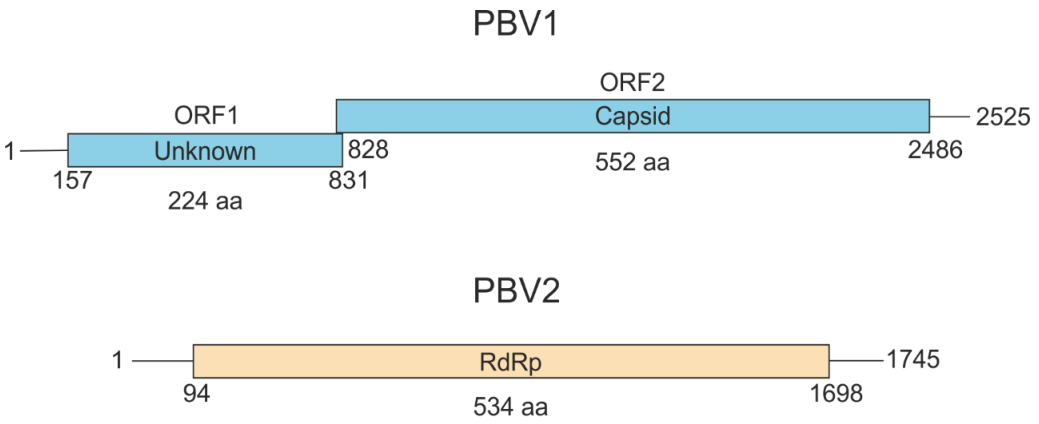

Figure 1. The human picobirnavirus (hPBV) genome organization. ORF: open reading frame, aa: amino acid, RdRp: RNA-dependent RNA polymerase. Modified from Wakuda et al., 2005.

1.2.2. *Cystoviridae*

The *Cystoviridae* family contains only one genus: *Cystovirus* (Poranen and Mäntynen, 2017). Cystoviruses are so far the only known bacteriophages (i.e. bacteria infecting viruses) which have a dsRNA genome. Their natural hosts are *Pseudomonas* species, like *Pseudomonas syringae*, which infects a wide range of plants in the temperate zone. The type species of the family is *Pseudomonas virus phi6* which was discovered 1973 and was for a long time the only member of this family (Vidaver et al., 1973). In the 1990s, eight new isolates, phi7–phi14 (Mindich et al., 1999), and ten years later a ninth isolate phi2954 (Qiao et al., 2010) were found. In 2015, PhiNN phage was isolated from lake water, in

Jyväskylä (Mäntynen et al., 2015). PhiNN, phi7, phi9, phi10 and phi11 are more closely related to phi6, based on sequence similarity, than other cystovirus species (Mindich et al., 1999), phiNN is the closest relative to phi6 (Mäntynen et al., 2015). A year later, the first cystovirus, PhiYY, infecting *Pseudomonas aeruginosa* was discovered from a clinical sample (Yang et al., 2016).

Phi6 has been discovered and studied since the 1970s (Vidaver et al. 1973) and is currently the best known cystovirus. It is composed of three concentric layers (Table 1; Fig. 2). The innermost core is called the polymerase complex (PC) and is composed of four structural proteins: P1, P2, P4 and P7 (Bamford and Mindich, 1980; Ktistakis and Lang, 1987; Mindich and Davidoff-Abelson, 1980; Olkkonen and Bamford, 1987). The major capsid protein P1 forms 60 asymmetric dimers from 120 copies of P1 which form triangulation number T=1 lattice (Butcher et al., 1997; Huiskonen et al., 2006; Ktistakis and Lang, 1987; Olkkonen and Bamford, 1987). Approximately 10 copies of P2 RdRps responsible for the replication of the phi6 genome are located inside the P1 shell near the three-fold symmetry axes and 72 copies of the packaging NTPase P4 forming hexamers at the five-fold vertices of the viral capsid are responsible for the packaging of the ssRNA genomic precursors (Butcher et al., 1997; de Haas et al., 1999; Ilca et al., 2015; Nemecek et al., 2012; Sen et al., 2008; Sun et al., 2012). Protein P7 is an assembly factor facilitating the PC assembly and RNA packaging located around the three-fold axes inside the capsid (Nemecek et al., 2012; Poranen et al., 2001). The innermost parts of phi6 have clear structural similarities with the eukaryotic reoviruses (Bamford et al., 2002). In fact, phi6 is an important model for the assembly of dsRNA viruses since the inner parts can also be assembled *in vitro* from the protein components (Poranen et al., 2001; Sun et al., 2012, 2014; Sun et al., 2013). The middle layer of the virion is formed by the nucleocapsid surface shell protein P8 arranged in T=13 icosahedral symmetry (Bamford and Mindich,

1980; Butcher et al., 1997; Etten et al., 1976; Sun et al., 2017). The outermost layer of the virus is a lipid-protein envelope (Etten et al., 1976). It is composed of five membrane proteins, P3, P6, P9, P10 and P13, of which P3 is peripheral and all the others are integral membrane proteins and host derived phospholipids (Etten et al., 1976; Gottlieb et al., 1988; Laurinavicius et al., 2004; Sinclair et al., 1975; Stitt and Mindich, 1983b). P3 together with the fusogenic protein P6 form a spike complex which attaches to the host cell pilus in the early stages of the viral life cycle (Bamford et al., 1976; Stitt and Mindich, 1983b).

The phi6 genome is located inside the viral PC and is composed of three dsRNA genome segments small (S), medium (M) and large (L) (Fig. 3) (Semancik et al.,

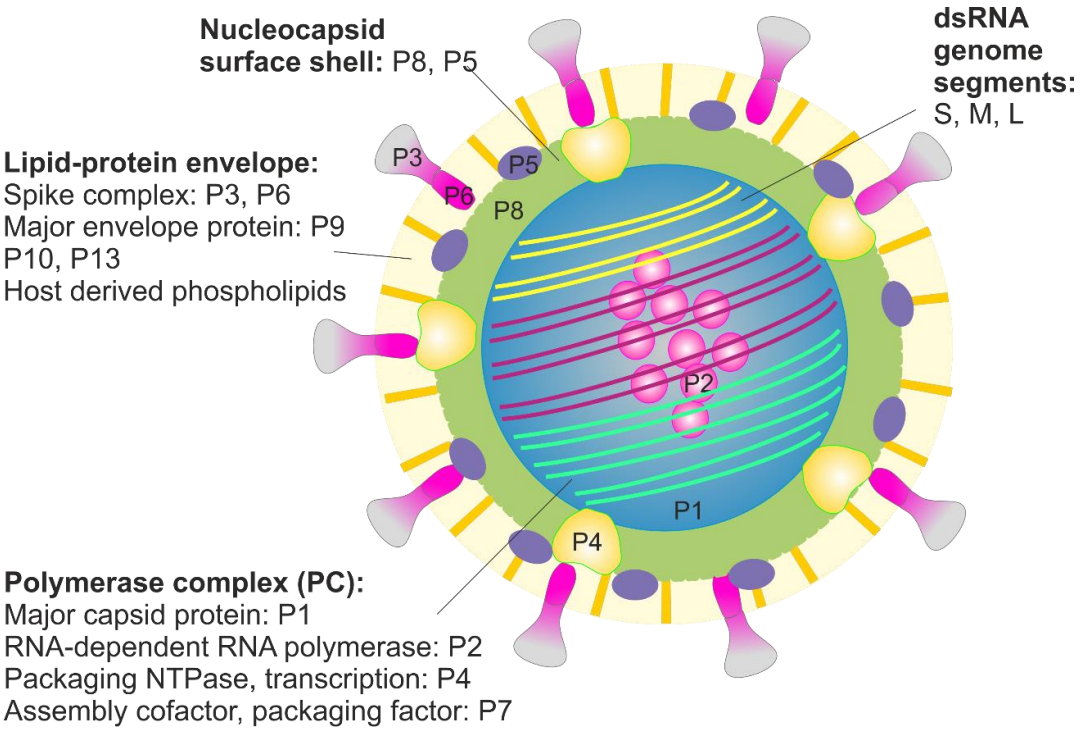

Figure 2. **The structure of bacteriophage phi6.** The polymerase complex (PC) is composed of proteins P1, P2, P4 and P7, the nucleocapsid surface shell of P8 and P5 and the envelope of proteins P3, P6, P9, P10, P13 and phospholipids. Modified from Oksanen *et al*., 2010.

1973; Van Etten et al., 1973). One copy of each segment is packaged into each virion and the total size of the phi6 genome is 13.4 kbp (Day and Mindich, 1980). The L-segment (6374 bp) encodes all the proteins needed for the PC formation, P1, P2, P4, and P7, and one non-structural protein P14 (Casini and Revel, 1994; Frilander et al., 1995). The nucleocapsid surface shell proteins P8 and P5 are encoded by the S-segment (2948 bp) and the proteins needed for the envelope assembly by the S- and M-segment (4063 bp) (Gottlieb et al., 1988; McGraw et al., 1986). This genomic organizations is well conserved among all the known cystoviruses (Mäntynen et al., 2015; Yang et al., 2016). The structure and organization of the phi6 dsRNA genome has been recently solved (Ilca et al., 2019). The genomic RNA is packed inside the capsid in a single-spooled manner, similar to that predicted for double-stranded (ds)DNA viruses and can also have slightly different conformations (Ilca et al., 2019).

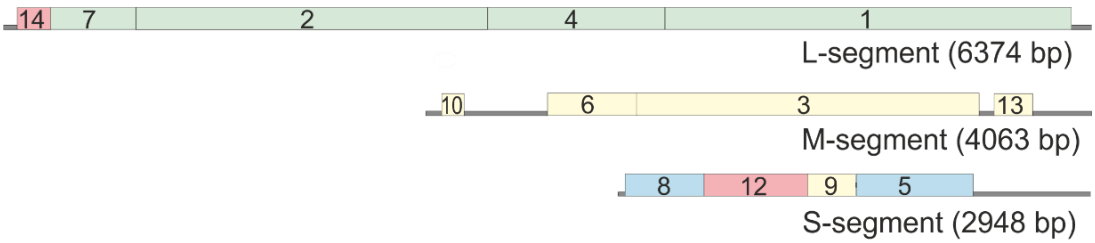

Figure 3. The phi6 genomic organization.
Modified from Bamford and Poranen, 2012.

1.3. Assembly of dsRNA viruses

All dsRNA viruses forming an icosahedral protein capsid (see exceptions from 1.2) replicate and transcribe their genome within this capsid structure, the PC. Non-infected cells do not normally possess long dsRNA. When dsRNA is recognized by the cell a cascade starts to defend it against the invading organism (Gantier and Williams, 2007). The viral capsid protects the genomic dsRNA from this cellular defense mechanism since the dsRNA always stays inside the capsid and never enters the cytoplasm. The genomic RNA is always encapsidated into the viral protein capsid in single-stranded form. However, for protein capsid assembly there are several different strategies. CPs can form an empty protein shell, a procapsid, where the single-stranded precursor genome is packaged in an energy-dependent process. This genome encapsidation pathway is common among the dsDNA bacteriophages, but cystoviruses are the only dsRNA viruses known to package their genome actively into preformed procapsids (Frilander and Bamford, 1995; Gottlieb et al., 1991; Hanhijarvi et al., 2017). Other dsRNA viruses use the co-assembly of CPs and ssRNA molecules by protein-protein and protein-nucleic acid interactions to form their capsid structures (Borodavka et al., 2018). To ensure that sufficient number of RdRp molecules are packaged inside the newly formed capsid, dsRNA viruses have also developed different strategies. Totiviruses produce, in addition to single CPs, CP-RdRp fusion proteins which co-assemble together with single CPs to form the virus (Dinman et al., 1991; Icho and Wickner, 1989). Pseudomonas phage phi8, a cystovirus and phi6 relative, nucleates the procapsid assembly using its RdRp ensuring that RdRps are always packaged to the capsid. During rotavirus infection the viral RdRp binds to the RNA-capping protein and this complex binds to ssRNA genomic precursors and interacts with the CPs. Thus, the capsid is formed and ssRNAs packaged simultaneously (Trask et al., 2012). When the viral capsid has

been formed and the genomic precursor ssRNAs packaged, the polymerase replicates the ssRNAs to form the dsRNA genome. We shall go more in detail the lifecycle and assembly of Pseudomonas phage phi6 below (1.3.1. – 1.3.4.). The human picobirnavirus lifecycle and assembly process are largely uncharacterized.

1.3.1. Cystovirus phi6 lifecycle

Pseudomonas phage phi6 uses the spike complex, composed of proteins P6 and P3, to attach to the host cell pilus (Fig. 4.A) (Stitt and Mindich, 1983b). When the pilus retracts it brings the virus next to the OM and the viral envelope and the host OM fuse due to the action of fusogenic P6 protein (Fig. 4.B) (Bamford et al., 1987). Most of the other cystoviruses use the lipopolysaccharides (LPS) of the host as their primary receptors (Mindich et al., 1999; Qiao et al., 2000). After the fusion of the membranes, muramidase (lysozyme) enzyme, P5, digests the peptidoglycan layer of the host (Fig. 4.C) and the nucleocapsid enters the cell endocytically through the plasma membrane by the help of P8 protein (Fig. 4.D) (Caldentey and Bamford, 1992; Mindich and Lehman, 1979; Poranen et al., 1999; Romantschuk et al., 1988). Inside the host the virion loses the plasma membrane and nucleocapsid surface shell (P8) by an unknown mechanism (Fig. 4.E) followed by the early transcription of the three precursor genomic segments s, m and l (Fig. 4.F) (Kakitani et al., 1980). From the transcribed positive sense l-segment the PC proteins, P1, P2, P4 and P7, are translated (Fig. 4.G) and from them the empty PCs or procapsids are formed (Fig. 4.H) (Bamford and Mindich, 1980; Mindich and Davidoff-Abelson, 1980). The empty PC packages the ssRNA genomic precursors (Fig. 4.I) and after all the segments s, m, l have been packaged the positive sense ssRNA genomes are replicated and finally the late transcription begins (Fig. 4.J) (Frilander and Bamford, 1995; Qiao et al., 1995).

From the late transcribed genome segments m and s, nucleocapsid surface shell and envelope proteins are translated (Rimon and Haselkorn, 1978). First, the P8 shell is formed around the expanded genome containing PC thus forming the nucleocapsid (Fig. 4.K) (Bamford and Mindich, 1980; Olkkonen et al., 1991). Finally, the lipid-protein envelope is formed around the nucleocapsid in the middle parts of the host cell (Fig. 4.L) (Bamford et al., 1976). The ready virions are released by host cell lysis (Fig. 4.M) (Mindich and Lehman, 1979; Vidaver et al., 1973).

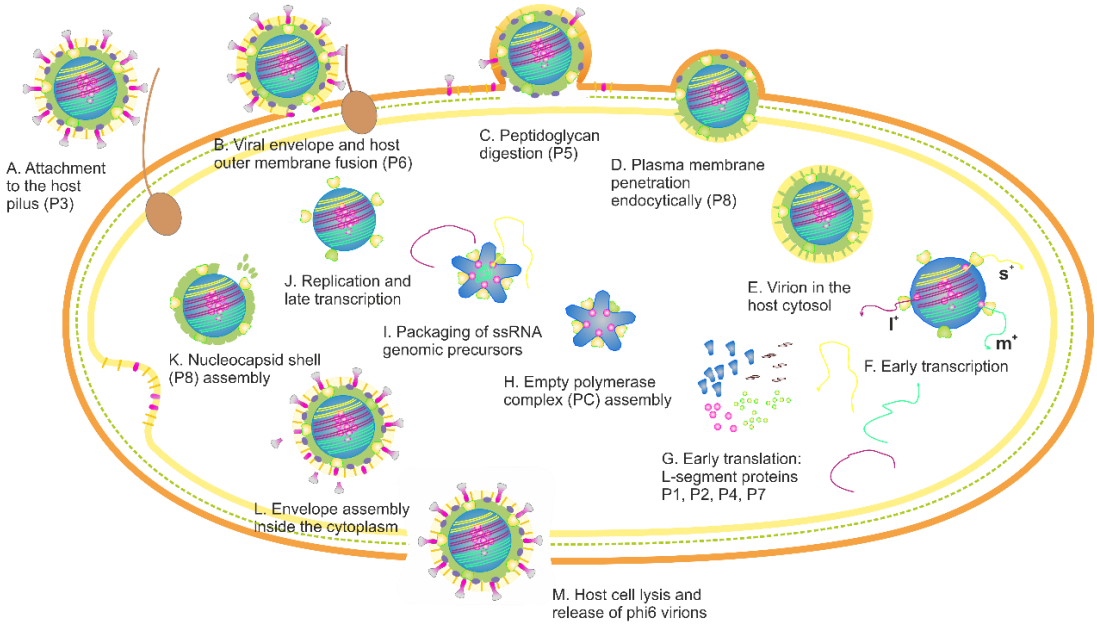

Figure 4. The phi6 lifecycle.
Modified from Oksanen *et al.*, 2010.

1.3.2. Assembly of the phi6 nucleocapsid

The assembly of the phi6 capsid starts when the early transcription and translation have taken place and the procapsid proteins, P1, P2, P4 and P7 form the empty precursor procapsid. Six P4 monomers form a hexamer in NTP and

magnesium-dependent reaction (Juuti et al., 1998). These P4-hexamers nucleate the assembly together with major capsid protein P1 (Poranen et al., 2001). After the nucleation process, more P4-hexamers and minor capsid proteins P7 are recruited to stabilize the nucleation complex (Juuti and Bamford, 1997). Finally, the individual CPs are added and the procapsid is formed. Incorporation of P2 RdRp into the procapsids is kinetically favored and depends on specific interactions with P1 (Ilca et al., 2015; Sun et al., 2018). The precursor genomic segments s, m, and l are packaged by P4 (Frilander and Bamford, 1995; Pirttimaa et al., 2002) and once all the segments are packaged and the ssRNA replicated to dsRNA by the RdRp P2 the procapsid undergoes conformational changes and expands to mature, dsRNA filled PC (Butcher et al., 1997). After the PC assembly has been completed the nucleocapsid surface shell is formed by protein P8, which is encoded by the S-segment (McGraw et al., 1986). The formed double-layered particle is called nucleocapsid (Etten et al., 1976).

1.3.3. Phi6 envelope assembly

The final step of the phi6 maturation is the envelope assembly which takes place in the central parts of the host cell cytosol (Bamford et al., 1976). This envelope formation strategy is rather unique among viruses since most of the enveloped animal viruses acquire their envelope by budding through the host cell cytoplasmic membrane (CM). Although, the assembly of the phi6 PC and the nucleocapsid surface shell have been thoroughly studied due to the elaborate *in vitro* systems (Ojala et al., 1990; Olkkonen et al., 1991; Poranen et al., 2001; Sun et al., 2012, 2014; Sun et al., 2013), the envelope assembly process is still largely uncharacterized and no *in vitro* system exists for studying the envelopment of the virus (Ojala et al., 1990; Poranen et al., 2001). The current knowledge about the phi6 envelope formation is based on phi6-infected cells, studies on mutant

viruses and expression of viral membrane proteins in different expression systems (Johnson and Mindich, 1994; Mindich and Lehman, 1983; Mindich et al., 1976; Sarin et al., 2012). Already in the 1970s, Mindich, *et al*. detected in their mutant studies, that if the phi6 major envelope protein P9 was not expressed in the infected cells, the phi6 particles lacked the lipid envelope and all the other membrane proteins (P3, P6, P10, P11, and P13) (Mindich et al., 1976). The resulting particles were similar when P12 was not expressed (Mindich et al., 1976). Thus, they concluded that the major envelope protein P9 and the non-structural protein P12 are essential in the phi6 virion envelopment (Mindich et al., 1976). These proteins are encoded consecutively by the S-segment of the virus (Fig. 3) (McGraw et al., 1986). This genomic organization is conserved among all sequenced cystoviruses also indicating an important role for these proteins (Mäntynen et al., 2018).

The major envelope protein P9 is a small, 9.5 kDa protein with a 16 hydrophobic aas in its N-terminus and a putative transmembrane region between 51 – 66 aas (McGraw et al., 1986). The hydrophobic N-terminus often indicates a signal sequence in this region. However, in P9 there is no signal sequence and only the outermost methionine at the N-terminus is cleaved after translation (McGraw et al., 1986). P9 is an integral membrane protein and most probably attached to the CM via its transmembrane region during the natural phi6 infection. It has been used as a fusion partner to facilitate the expression of eukaryotic membrane proteins in bacteria. The P9-tag both enhances the expression and the integration into the *E. coli* CM (Jung et al., 2015). The non-structural protein P12 is also a relatively small, 20.3 kDa, and 195 amino acid protein. There have been several hypotheses of the functional mechanisms of P12. Johnson and Mindich observed that although P12 was needed to detect P9 in their expression system, it was not needed in the translation of P9 (Johnson and Mindich, 1994). They postulated

that P12 might act as a protease inhibitor since it stabilizes P9 (Johnson and Mindich, 1994). In addition, it has been suggested that P12 would assist all the membrane proteins to correct pathways or act as a lipid-transfer protein transferring the phospholipids from the host CM onto the nucleocapsid (Mindich and Lehman, 1983; Stitt and Mindich, 1983a).

1.3.4. Biotechnological potential of lipid vesicles

The potential of biological vesicles in biotechnology and biomedicine was recognized already in 1960s (Bangham, 1978; Bangham et al., 1962; Bangham and Horne, 1964). Due to the aqueous interior surrounded by a lipid bilayer, vesicles can carry both hydrophilic and hydrophobic molecules (Drulis-Kawa and Dorotkiewicz-Jach, 2010). Synthetic vesicles have been used in biomedicine to enhance the efficiency of antibiotics (Drulis-Kawa and Dorotkiewicz-Jach, 2010), to target drugs specifically to certain cells, for instance to cancer cells (Kao et al., 1996), and for genetherapy treatments (Zylberberg et al., 2017). Phi6 bacteriophage provides an attracting tool for membrane research and vesicle production due to its ease of cultivation and relatively simple structure. In addition, it has been shown that phi6-specific membrane vesicles can be produced in *E. coli* by expressing P9, P12 and P8 proteins (Sarin et al., 2012), which allows the production of vesicles without the vesicle background of eukaryotic cells. Recently, phi6-specific vesicles were used as synthetic lipid-containing scaffolds in enhancement of the production of indigo in *E. coli* (Myhrvold et al., 2016). In this system, expression of P9 and P12 produced lipid-scaffolds, which co-localized the enzymes needed in the indigo production and enhanced the indigo yield by 2–3 fold (Myhrvold et al., 2016).

1.4. RNA replication by dsRNA viruses

RNA viruses can proliferate fast and their replication is error prone (Domingo and Holland, 1997). Thus, all RNA viruses, including dsRNA viruses, are capable of fast evolution (Domingo and Holland, 1997). All RNA viruses need their own polymerase since host cells do not support RNA synthesis from an RNA template. These polymerases synthesize RNA in 5' – 3' direction from a RNA template.

1.4.1. RNA-dependent RNA polymerases

All known RdRps from dsRNA viruses have a right-handed core polymerase domain structure. A high-resolution structure has been determined for seven dsRNA virus RdRps. Mammalian reovirus (Tao et al., 2002), rotavirus (Lu et al., 2008) and cypovirus 1 (Zhang et al., 2015) polymerases possess a cage-like structure with four channels leading to the active, inner parts of the enzyme. Cystovirus, phi6 (Butcher et al., 2001; Salgado et al., 2004) and phi12 (Ren et al., 2013), and birnavirus, infectious bursal disease virus (Graham et al., 2011) and infectious pancreatic necrosis virus (Garriga et al., 2007), polymerases are smaller than reovirus polymerases and contain only three channels to the active site (see 1.3.3. Phi6 RdRp). The classical signature amino acid sequence of RdRps is GDD (glycine, 2×aspartic acid) (Kamer and Argos, 1984; Koonin et al., 1989). This sequence is changed to SDD (serine, 2×aspartic acid) in phi6 RdRp (Mindich et al., 1988). This RdRp motif is in the active site of the polymerase where the actual RNA synthesis happens. We shall now go through different biochemical activities of viral RdRps and the structure, function and biotechnological applications of the phi6 RdRp.

1.4.2. Initiation modes of RNA-dependent RNA synthesis

There are two principal initiation modes for RNA-dependent RNA synthesis into which all different ways of RNA-dependent RNA synthesis can be grouped: primer-independent and primer-dependent initiation (van Dijk et al., 2004). In the primer-independent initiation mechanism the synthesis of RNA is started without a primer i.e. *de novo*. For the *de novo* initiation complex to form and the initiation to occur the RdRp, NTPs, divalent cations and the template are required. The first appropriate incoming nucleotide acts as a primer and the second nucleotide is attached to the 3'OH group of the first nucleotide by a phosphodiester bond. The *de novo* initiation mechanism of phi6 RdRp is presented more in detail in the section 1.4.5 Phi6 RNA-dependent RNA polymerase.

In the primer-dependent initiation mechanism an oligonucleotide or a protein primer is needed for the formation of the initiation complex. The oligonucleotides can be derived from different sources. Some (-)ssRNA viruses use oligonucleotides cleaved from the 5'capped ends of the host mRNAs. This mechanism is known as "cap-snatching" and has been detected for e.g. influenza A virus (De Vlugt et al., 2018). Another way of producing nucleotide primers is abortive initiation which is used by rotavirus (Chen and Patton, 2000). In this method the polymerase starts RNA synthesis *de novo* several times to produce short dinucleotides which are eventually used as primers (Chen and Patton, 2000). Picornaviruses (poliovirus) and some caliciviruses (norovirus) use protein primer in the RNA synthesis initiation (Paul et al., 1998; Rohayem et al., 2006b). The priming protein VPg will remain covalently attached to the 5'-end of the positive-sense genomic ssRNA after the viral replication (Paul et al., 1998). The OH-group of the VPg is uridylylated and then serves as a primer for the first

NTPs to be linked in the RNA synthesis (Paul et al., 1998). One way of priming the RNA synthesis is back-priming or template-primed initiation in which the 3'-end of a template bends backwards and the 3' terminal OH-group serves as a primer for the new RNA strand (I, Fig. 4a). This method is usually fatal to the virus since the genomic strands are linked together covalently and the following replication rounds cannot be achieved if back-priming has occurred (Laurila et al., 2005; Noton et al., 2014). Thus, it is avoided in the natural infection of the RNA viruses.

1.4.3. Replication and transcription of dsRNA viruses

Replication activity of RdRp means the synthesis of a complementing strand to the ssRNA template. In the case of dsRNA viruses replication happens after all the positive-sense ssRNA genomic precursors have been packaged and means the synthesis of the minus-strand.

Transcription activity means the synthesis of single-stranded positive-sense RNA from the dsRNA template which can be used as a template for the translation of viral proteins. DsRNA viruses can have two mechanisms for transcription: conservative or semi-conservative transcription mechanism (I, Fig. 5a). In the conservative transcription the newly produced transcript is dissociated from the duplex and as an end product in this process there will be newly produced daughter transcripts. If there are for example radioactively labeled nucleotides available, these daughter transcripts will be radioactively labeled (I, Fig. 5a). In the semi-conservative transcription the newly produced transcript displaces the parental positive-strand and the end product will be a parental positive-sense ssRNA transcript and, the double-stranded template RNA molecules are labeled if there are radioactive nucleotides available (I, Fig. 5a).

Processivity of an RdRp means the ability of the enzyme to elongate the RNA production after the initiation of the RNA synthesis (Breyer and Matthews, 2001). It is one of the key features of a polymerase for obvious reasons: if the polymerase does not have an efficient enough processivity the nucleic acid of the organism will not be fully replicated and instead only different sizes of replication and transcription products are produced (Breyer and Matthews, 2001).

1.4.4. Terminal nucleotidyl transferase activity

Terminal nucleotidyl transferases (TNTases) are enzymes that catalyze the addition of one or several nucleotides to the 3'-end of nucleic acid molecule, DNA or RNA, without template. Cellular TNTases have several recognized functions the most well-known being probably the addition of a poly-A-tail to the 3'-end of mRNA (Stewart, 2019). Nevertheless, the functions of the viral TNTases in the viral lifecycles remain unclear. Several ssRNA virus polymerases have TNTase activity in addition to their normal replicase and transcriptase activities, e.g. poliovirus and hepatitis C virus (HCV) (Behrens et al., 1996; Neufeld et al., 1994). Among dsRNA virus polymerases only phi6 polymerase is known to possess TNTase activity (Poranen et al., 2008a).

1.4.5. Phi6 RNA-dependent RNA polymerase

RNA-dependent RNA polymerase activity of Pseudomonas phage phi6 has been specified to the P2 protein (Makeyev and Bamford, 2000). P2 is encoded by the L-segment from which also the other three protein components of the polymerase complex are encoded (Fig. 3) (Mindich et al., 1988). It has 664 amino acids and is the second largest, 74.8 kDa, of the proteins of phi6 measured by the molecular weight (Mindich et al., 1988). The structure of P2 has been solved to 2.0 Å resolution (Butcher et al., 2001). It has a canonical right-handed polymerase

shape with palm, fingers and thumb subdomains (Butcher et al., 2001). Phi6 polymerase resembles closely reovirus RdRps, phage Qβ RdRp and HCV and other flavivirus polymerases (Butcher et al., 2001; Mönttinen et al., 2014). Thus there is an interesting evolutionary link between (+)ssRNA flaviviruses and dsRNA viruses (Butcher et al., 2001; Mönttinen et al., 2014). The palm structure with the active site is the most conserved part of phi6 and HCV polymerases (Butcher et al., 2001). The active site is located in the middle of the relatively spherical protein. Two positively charged tunnels lead into the active site: the template tunnel and the substrate tunnel through which the ssRNA template and the NTPs are transported, respectively. In the active site, three conserved aspartic acid residues (D324, D453, and D454) coordinate one manganese and two magnesium ions in order to form the initiation complex (Butcher et al., 2001). In addition to these conserved structures, phi6 polymerase has fingers strapped to the thumb by six polypeptide strains, the so called finger tips (Butcher et al., 2001). In the C-terminus of the polymerase, there is a special structure of 64 amino acid residues which enables the *de novo* initiation of the RNA synthesis (Butcher et al., 2001).

The formation of the initiation complex starts when the 3'-end of genomic ssRNA enters the template tunnel of the polymerase (Butcher et al., 2001; Salgado et al., 2004; Sarin et al., 2009). The first nucleotide from the 3'-end of the negative-strand of phi6 genome segments is cytosine (CTP) and is designated as T1. It is buried inside a C-terminal specificity pocket (S-pocket) of the polymerase leaving the second nucleotide of the template, T2, exposed in the active site (Butcher et al., 2001; Salgado et al., 2004). The first incoming NTP of the daughter strand, D2, is stabilized by the magnesium and manganese divalent cations (Mg^{2+}; Mn^{2+}) and is positioned into the active site by stacking interactions with tyrosine Y630 and by Watson-Crick pairing with the template

T2 nucleotide (Butcher et al., 2001; Poranen et al., 2008b). The T1 nucleotide is released from the S-pocket and paired with D1 nucleotide. The phosphodiester bond is formed between D1 and D2 in a reaction catalyzed by two Mg^{2+} ions and one pyrophosphate (PPi) is released with one Mg^{2+} ion. For RNA synthesis to continue the polymerase has to shift from the initiation to the elongation mode involving movement of the C-terminal domain (Wright et al., 2012). In this process the bound Mn^{2+} is released and must be replaced for elongation (Poranen et al., 2008b; Wright et al., 2012).

1.4.6. Phi6 RdRp based biotechnological applications

The phi6 RdRp can replicate ssRNA templates of different sizes and sequences from different organisms without aid of any other protein components. These features makes it an attractive biotechnological tool for enzymatic dsRNA production (Aalto et al., 2007). The produced dsRNA can be cleaved with enzymatic reactions to small interfering RNAs. Phi6 polymerase produced dsRNA and small interfering RNAs have been used to inhibit expression of a transgene in human cells (Aalto et al., 2007), as an antiviral against herpes simplex virus (Paavilainen et al., 2016, 2017; Paavilainen et al., 2015; Romanovskaya et al., 2012), and different influenza A virus strains (Jiang et al., 2019). DsRNA pools produced using phi6 RdRp have also been developed to efficient crop protectants (Niehl et al., 2018).

2. AIMS OF THE STUDY

The aim of this study was to analyze the molecular details of the genome replication and the assembly pathways of dsRNA viruses. Pseudomonas phage phi6 was selected to study the envelope biosynthesis as it is the only known bacterial virus with an enveloped nucleocapsid. Bacteriophage phi6 provides a tool to study membranous structure formation in bacterial cells. For the analysis of the dsRNA virus genome replication processes we selected human picobirnavirus as a model. Picobirnaviruses are a recently described group of viruses and no cell culture infection systems exists for the analysis of their replication mechanisms. Therefore, *in vitro* and recombinant protein expression based systems are the only option to study the lifecycle of this virus.

The specific aims of this study were:

1) To characterize the enzymatic activities of the hPBV RdRp and to investigate the incorporation of the RdRp into the viral capsid.

2) To determine the structure of the hPBV RdRp and to identify structural features essential for the *de novo* initiation of RNA synthesis.

3) To determine the minimum requirements for phi6-specific vesicle formation and the hPBV capsid assembly.

4) To analyze conditions required for the addition of heterologous proteins to the phi6-specific vesicles in *Escherichia coli*.

5) To gain new insights to the envelope assembly of the Pseudomonas phage phi6 and the functions of the essential membrane-assembly proteins P9 and P12.

3. MATERIALS AND METHODS

Escherichia coli JM109 (Yanisch-Perron et al., 1985) was used as a cloning strain and *E. coli* BL21(DE3) (Studier and Moffatt, 1986) and Rosetta 2 (Novagen) for the expression of recombinant proteins. Cells were cultivated in L-broth [1% (w/v) Bacto tryptone, 0.5% (w/v) Bacto yeast, and 0.5% (w/v) sodium chloride], at 37°C at 200 rpm shaking, unless otherwise stated. Selected protein coding sequences were cloned in plasmids pET28a(+), pET28b(+), and pCDF-1b (Novagen) used as expression vectors. The expression plasmids derived from these vectors are presented in II, Table 1. The plasmids used for ssRNA production were based on pT7T319U and pMA-RQ vectors and the derivatives are presented in I, Table S2. Nucleotide sequence data was obtained from GenBank.

The methods used in this study are summarized in Table 2 and described in detail in the relevant articles I and II.

Table 2. The methods used in this Thesis	
Method	**Used in**
Agarose gel electrophoresis	I, II
Complementary DNA production by polymerase chain reaction	I
Coomassie blue staining of proteins	II
Density determination of the flotation fractions by weighting	II
Detection of light-scattering and green fluorescent bands using visible light	I, II
Determination of crystal structures by single-wavelength anomalous dispersion	I
dsRNA production using phi6 RdRp	I
dsRNA purification by stepwise lithium chloride precipitation	I
Expression of recombinant proteins in *Escherichia coli*	I, II
Flotation ultracentrifugation	II
Fluorescence microscopy	II
Gel filtration chromatography	I
Gel shift assays	I
Genomic phi6 dsRNA purification	I
Glutaraldehyde fixation of *E. coli* cells	II
Immobilized metal ion (nickel) affinity chromatography	I
In vitro RNA production assays with radioactively labeled nucleotides	I
Molecular cloning/subcloning	I, II
Negative staining of electron microscopy samples with uranyl acetate	I, II
Polyethylene glycol precipitation of vesicles	II
Protein crystallization	I
Quantitative analysis of EM thin sections using Aida Image Analyzer	II
Rate-zonal ultracentrifugation	I, II
Site-directed mutagenesis	I
Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)	I, II
ssRNA production using T7 polymerase	I
ssRNA purification by chloroform extraction and lithium chloride precipitation	I
Sudan Black staining of proteins	II
Trichloroacetic acid precipitation of flotation centrifugation fractions	II
Transmission electron microscopy	I, II
Western blotting	I, II

4. RESULTS AND DISCUSSION

4.1. Genome replication of a small dsRNA virus

4.1.1. Enzymatic activities of the hPBV polymerase

The hPBV polymerase activities were studied using wild type (wt) RdRp and a derivative in which residues 495–518 (forming the insertion loop; see 4.1.2.) were deleted (Δloop). Both polymerases could replicate positive-strand and negative-strand homologous picobirnaviral ssRNA and positive-strand heterologous phi6 ssRNA *in vitro* in the absence of the CP (I, Fig. 3b). Also, phi6 RdRp can replicate and transcribe in the absence of the other PC proteins, whereas, most dsRNA virus polymerases are active only in the presence of the CPs. This ensures that the polymerase will only replicate encapsidated virus-specific RNA as outside the capsid the possibility of host mRNA replication exists. In addition to the full-length dsRNA, the wt hPBV polymerase produced also shorter dsRNA from the phi6 ssRNA. This phenomenon means that it had some processivity issues with the heterologous RNA (I, Fig. 3b, left panel).

The mechanism of dsRNA transcription was studied by *in vitro* transcription assays. Unlabeled dsRNA substrates were radioactively labeled, thus, indicating that the hPBV polymerase uses semi-conservative transcription mechanism (I, Fig. 3b, Fig. 5). In addition, the results from the time-course experiment supported the semi-conservative mechanism since the dsRNA molecules were radioactively labeled before the ssRNA transcripts (I, Fig. S4). Both wt and Δloop hPBV polymerases were able to use PBV genome segment 2 (PBV2) and phi6 genomic dsRNA as templates for transcription, although, the transcription activity was much lower for the Δloop than for the wt polymerase (I, Fig. 3b).

Our result supports the idea that small RdRps with the three-tunnel system (phi6 (Butcher et al., 2001; Usala et al., 1980), birnavirus (Graham et al., 2011; Pan et al., 2007), and hPBV [I]) use the semi-conservative transcription, whereas, the large viral RdRps with four-tunnel system (rotavirus (Zhang et al., 2015), reovirus (Skehel and Joklik, 1969; Tao et al., 2002)) use the conservative transcription mechanism.

Interestingly, the hPBV polymerase had preference for the dsRNA molecules which have CAU-3' at the 3'-end of the negative-strand over those which have a CCU-3'-end (I, Fig. 5b, and 5c). This preference is logical since both genomic segments of hPBV start with 5'-GUA which is complementary to the preferred CAU-3'. Also, phi6 L-segment begins with the 5'-GUA explaining why hPBV RdRp prefers L-segment over the S- and M-segments of phi6 (I, Fig. 3b and 5b). Taken also into account that we have not detected any preference of the RdRp to a specific ssRNA template (I, Fig. 3b and S6A) we suggest that the hPBV RdRp preference for CAU-3'-end in dsRNA is due to the lower melting temperature of this sequence compared than the melting temperature of the CCU-3'. In addition, since the 3'-ends of the positive-strands are similar between phi6 genomic segments (I, Table S1), our data suggests that hPBV RdRp mainly uses the negative-strand as transcription template producing positive-strand ssRNAs transcripts.

We also detected terminal nucleotidyl transferase (TNTase) activity in the wt and Δloop hPBV polymerases (I, Fig. 3c and 3d). DsRNA and ssRNA were both used as substrates for this reaction but ssRNA was strongly preferred by both polymerases (I, Fig. 3d). The Δloop RdRp had stronger TNTase activity than the wt (I, Fig. 3d). It has been proposed previously that for the TNTase reaction the RNA substrate enters via the tunnel used for dsRNA exit during replication and

transcription reactions to orientate the 3'OH group for the nucleotide addition (Poranen et al., 2008a). When the C-terminal loop is missing, the exit site of the polymerase is open and the 3'-end can reach the active site more easily for the nucleotidyl transfer. Several viral polymerases of (+)ssRNA viruses have been detected to have TNTase activity, like hepatitis C virus, bovine viral diarrhea virus, norovirus and poliovirus (Behrens et al., 1996; Neufeld et al., 1994; Ranjith-Kumar et al., 2001; Rohayem et al., 2006a; Zhong et al., 1998). However, this is only the second reported dsRNA virus polymerase that has TNTase activity, phi6 polymerase was the first (Poranen et al., 2008a). The biological significance of the TNTase activity is still unclear.

4.1.2. Structure of the hPBV polymerase

In addition to the description of the hPBV RdRp enzymatic activities, in this Thesis we obtained new information about the structure of the RdRp. The structure of the hPBV RdRp was solved at 2.4 ångström (Å) resolution in its apo form (I, Fig. 1). Human PBV polymerase is oval shaped and a rather small polymerase (diameters ~50 × 60 × 60 $Å^3$). It shares with all the other known viral RdRps the canonical cupped right-hand shape structure (Mönttinen et al., 2014). The structure can be divided into three different domains: The N-terminal domain (1–84 aa), the core polymerase domain (85–470 aa) and the C-terminal domain (471–534 aa) (I, Fig. 1a). The core polymerase domain contains three subdomains: the palm (231–267, 325–414 aa), the thumb (415–470 aa) and the fingers (85–230, 268–324 aa) (I, Fig. 1b). The palm subdomain contains the conserved RdRp active site aspartic acids D261, D359, and D360. Three tunnels lead to the active site in the interior of the polymerase: the template tunnel for the ssRNA template, the substrate tunnel for the nucleoside triphosphates (NTPs) and the product tunnel for the nascent dsRNA (I, Fig. 2). This three-tunneled

structure largely resembles the structure of other small viral polymerases like phi6 and birnavirus RdRps (Butcher et al., 2001; Pan et al., 2007).

The hPBV polymerase has a flexible area near the C-terminus: the insertion loop structure (495–518 aa). This 24-amino acid structure extends towards the active site and its location is similar to the initiation platform of phi6 and flavivirus RdRps (Laurila et al., 2002; Laurila et al., 2005; O'Farrell et al., 2003). The general structure of the Δloop is similar to the structure of the wt polymerase except the C-terminal insertion loop is missing (I, Fig. 3a). This suggests that the deletion of the loop structure does not affect the overall folding of the polymerase and Δloop polymerase can be used to study the functions of the insertion loop of the hPBV polymerase.

4.1.3. The role of the insertion loop

We investigated the role of the C-terminal 24-aa loop structure (see 4.1.1.) by comparing the wt and Δloop hPBV RdRps and the mechanisms the RdRps use for the initiation of RNA replication. This ability was studied using ssRNA templates, and heat-denaturizing the reaction products before analysis by agarose gel electrophoresis (I, Fig. 4). Heat-denaturized replication products of the wt polymerase had similar mobility as the ssRNA template indicating that the produced dsRNA was composed of two complementary ssRNA molecules (I, Fig. 4b, left). However, the heat-denatured replication products of the Δloop polymerase had slower mobility in agarose gel than the template ssRNA. This indicates that the template strand and the produced complementary strand are covalently linked which prevents their dissociation to separate ssRNA molecules (I, Fig. 4b, right). Thus we concluded, that the wt polymerase used *de novo* initiation and the Δloop polymerase back-priming mode for the replication. To

confirm that the actual *de novo* initiation took place we repeated the replication experiment with gamma-labeled guanosine triphosphate ([γ-^{32}P]-GTP). In [γ-^{32}P]-GTP, the phosphorus of the third phosphate group (γ) of the GTP is radioactive P-32 (I, Fig. 4). During the RNA synthesis the two outermost phosphate groups (γ and β) are removed from the nucleotide and only the α-phosphate is left as the phosphodiester bond is formed. When the polymerase uses *de novo* initiation the radioactive label of [γ-^{32}P]-GTP stays attached to the 5'-end of the RNA molecule, whereas, if initiation happens via back-priming the radioactive label is cleaved off. Our results suggested that the insertion loop is needed in the *de novo* initiation of RNA synthesis most probably serving as an initiation platform similar to the phi6 polymerase initiation platform (Laurila et al., 2002; Laurila et al., 2005).

4.2. Assembly of dsRNA viruses

4.2.1. Human picobirnavirus assembly and RdRp encapsidation

Previously the only information on picobirnaviral capsid assembly has been from structural studies on the rabbit picobirnavirus (rPBV) (1.2.1 *Picobirnaviridae*) (Duquerroy et al., 2009). To better understand the human picobirnavirus assembly we expressed the CP alone in *E. coli* and together with the RdRp and investigated the resulting particles. Overexpression of only the CP led to the formation of VLPs with a diameter of about 35 nm (I, Fig. 6a) which is about the same size as the previously described for rPBV particles (Duquerroy et al., 2009). Thus, no other viral proteins are required for the assembly of the hPBV capsid suggesting that the CP can nucleate the self-assembly reaction. Rabbit PBV capsids contain proteolytically cleaved CP and it has been proposed that there is an autoproteolytic processing of the CP during the capsid assembly or maturation

(Duquerroy et al., 2009). To test whether hPBV CP is also proteolytically processed we over-expressed wt CP and Δ45 CP in which the first 45 N-terminal amino acids were deleted. The Δ45 CP also formed VLPs detectable in the electron microscopy (I, Fig. 6a) which indicates that the N-terminus of the CP is not required for the nucleation of hPBV capsid assembly or for making critical interactions within the capsid. When the VLPs were analyzed by SDS-PAGE the Δ45 CPs migrated faster than the wt CPs originating from the VLPs (I, Fig. 6b) indicating that, in these experimental conditions, wt hPBV CP does not undergo similar autoproteolytic cleavage as the rPBV CP (Duquerroy et al., 2009).

To investigate how the RdRps are packaged inside the hPBV capsids, we co-expressed CPs and histidine-tagged RdRps in the *E. coli* expression system. RdRps which were not packaged inside the VLPs were removed from the cell lysate by the nickel-column affinity chromatography. RdRps could not be detected in the purified VLPs although they were present in the clarified lysate (I, Fig. 6c). This result indicates that for the RdRp incorporation something else than just protein-protein interactions between the RdRps and CPs are needed. However, we cannot rule out the possibility that the N-terminal histidine-tag of the RdRp prevented critical RdRp-CP interactions necessary for RdRp packaging. Nevertheless, the result indicates that interactions between the RdRp and viral genomic ssRNA molecules could be essential for the encapsidation of the RdRp. Or more specifically, the interaction between the RdRp and the untranslated regions (UTRs) since only the coding RNA sequences were present in the co-assembly system. To test this hypothesis, we prepared binding assays using three different ssRNA oligonucleotides: non-specific CA-repeat, and 5'- and 3'-UTRs of the positive-strand of hPBV genome segment 2. We also used two longer PBV-specific ssRNAs: full-length positive-sense genome segment 2 (+PBV2) and +PBV2 with deletion in nucleotides 1-645 (Δ1-645). Wt RdRp had

10-fold higher affinity to the 5'-UTR than for the 3'-UTR or the non-specific CA-sequence and 10-fold higher affinity to the full-length than Δ1-645 +PBV2. The deletion of the insertion loop did not affect these results significantly. Since, RNA synthesis requires entry of the 3'-end of the positive-strand to the template tunnel, the binding of the polymerase to the 5'-UTR may indicate that this region assists the co-assembly of hPBV CPs, RdRps and genomic segments. The results with the Δloop RdRp were similar suggesting that the C-terminal priming loop is not involved in the binding of 5'-UTR. Another explanation for these results is that the hPBV RdRp affinity to the 5'-UTR may facilitate the entry of the 3'-end of the negative-strand to the template tunnel for transcription reaction and thus enhance the RNA synthesis.

Based on our data we propose a co-assembly model for hPBV. HPBV CPs can self-assemble to VLPs without any other viral components. Also many ssRNA virus CPs can self-assemble *in vitro*, but the capsid assembly is much more efficient when the ssRNA genome is present (Twarock et al., 2018). Specific stem-loop structures have been identified in the genomic RNA of several (+)ssRNA viruses, which bind to CPs and facilitate the assembly of the capsid (Dykeman et al., 2014; Patel et al., 2017; Rolfsson et al., 2016; Twarock et al., 2018). More experiments are needed to find the potential packaging signals in the hPBV genome which would recognize the RdRp and CPs to fulfill our co-assembly model.

4.2.2. Phi6 envelope assembly

Although the inner parts of the Pseudomonas phage phi6 are well studied (see 1.3.2 Assembly of the phi6 nucleocapsid), the assembly of the outermost layer, the envelope, is still largely uncharacterized. Our aim was to shed light on the envelope formation process and to determine the minimum requirements for the

membrane structure formation by expressing the phi6 membrane proteins in *E. coli*. In addition, our goal was to evaluate the possibility to produce bacterial membrane vesicles with heterologous proteins for biotechnological applications. We demonstrated that expression of the phi6 major envelope protein P9 resulted in the formation of membranous structure in the cytoplasm of *E. coli* (II, Fig. 1). The closer investigation of these membranous structures revealed that they floated higher in the flotation centrifugation than the CM and OM of *E. coli* and were highly P9-specific (II, Fig. 2a, c, d, and e). The density of the P9-specific vesicles was approximately 1.13 g/cm^3 (II, Fig. 2a) and they could be also visualized by transmission electron microscopy (II, Fig. 2b). Interestingly, these membranous structures and P9-vesicles were similar when the non-structural protein P12 was co-expressed with P9 (II, Fig. 1 and 2). In the flotation centrifugation analysis, P12 was associated with the CM fraction and not with the P9 vesicle fraction (II, Fig. 2d) suggesting that P12 acts in the cytoplasmic membrane.

We also tested whether heterologous green fluorescent protein (GFP) could be added to the P9-specific vesicles by expressing GFP with an N-terminally fused P9-tag (P9-GFP). When only P9-GPF was expressed in *E.coli*, green color was detected at the bottom of the flotation centrifugation tube and no P9-GFP vesicles could be detected (II, Fig. 3a). Western blot analysis revealed that, in fact, no full-sized fusion protein was present in the cell lysate and the non-floating green material represented most likely protease cleaved GFP-tag (II, Fig. 3b). The fate of the cleaved P9 is unclear. One possible option is that it might be folded incorrectly and also degraded by proteases. To test the hypothesis of P12 being a protease inhibitor (Johnson and Mindich, 1994), we added P12 to the expression system. Indeed, full-sized P9-GFP appeared when P12 was co-expressed with P9-GFP (II, Additional file 1). Nevertheless, green P9-GFP vesicles could be

only detected when both wt P9 and P12 were co-expressed with P9-GFP (II, Fig. 3a, and c). Although P9 was needed in the P9-GFP production, only the GFP-tagged version of the protein was detected in the P9-GFP-vesicle fraction. In the P9-GFP, P9 and P12 expression system, the less dense P9-vesicle fraction showed similar flotation pattern as observed earlier when P9 was expressed alone (II, Fig. 2a, and Fig. 3a). The P9-GFP vesicles were not just products of CM distribution since they could be purified and were specific to P9-GFP. Furthermore, the GFP signal was localized in the centrum of the *E. coli* cells (II, Fig. 3d, and e). One possible explanation to these findings is that the wt P9 is needed in the formation of membranous structures which are a pre-requisite for the formation of the P9-GFP vesicles. Our findings also support the hypothesis of Johnson and Mindich (Johnson and Mindich, 1994) that P12 may act as a protease inhibitor in the envelope assembly process of phi6.

5. CONCLUSIONS AND FUTURE PROSPECTS

In this Thesis the molecular details of the replication and assembly of two dsRNA viruses, Pseudomonas phage phi6 (phi6) and human picobirnavirus (hPBV), were investigated. Phi6 is a bacteriophage infecting *Pseudomonas* plant pathogens and hPBV has been associated with immunocompromised persons with gastroenteritis. Although, these viruses have different hosts and environmental requirements, they possess similar replication mechanisms. They both have relatively small, three-tunneled RdRps which are responsible for the replication and the transcription of the viral genome (I, Fig. 2) (Butcher et al., 2001). The overall structure of the polymerases is cupped right-handed structure similar to the other known viral RdRps (Mönttinen et al., 2014). Both have a C-terminal extrusion which acts as a priming platform allowing the *de novo* i.e. primer-independent initiation of the RNA synthesis (I, Fig. 4) (Butcher et al., 2001) and they both use a semi-conservative transcription mechanism (I, Fig. 3b and 5) (Usala et al., 1980). Since hPBV polymerase also uses the semi-conservative mechanism as other known small dsRNA virus RdRps (see 4.1.1.), our results brought more support to the idea that small, three-tunneled polymerases use semi-conservative, whereas, large, four-tunneled polymerases use conservative transcription. One of the most intriguing features of hPBV and phi6 polymerases is that they can replicate and transcribe heterologous templates and are enzymatically active in the absence of the capsid proteins making them interesting RNA production tools for the biotechnological applications. The biochemical potential of phi6 RdRp has already been demonstrated (Aalto et al., 2007; Niehl et al., 2018; Romanovskaya et al., 2012). The hPBV RdRp could be also used in dsRNA production due to its ability to replicate heterologous ssRNA in the absence of the CP. Although, the processivity issues should need more investigation before its wider use in biotechnology.

While the replication strategies of phi6 and hPBV are very similar, there are substantial differences in virus assembly mainly due to differences in virion structure and complexity of these viruses. Human PBV only has a single capsid protein and two dsRNA genome segments and the RdRp is located inside the capsid. The phi6 virion contains eleven structural proteins and has three structural layers (see 1.2.3. *Cystoviridae*). The two innermost ones are protein layers and the outermost a lipid-protein envelope without structural symmetry. The innermost protein layers of these viruses have structural similarities since both have T=1 structure where the asymmetric unit is a dimer, a typical structure to all dsRNA viruses. Nevertheless, the assembly strategy of the innermost capsid structure is different. Phi6 forms a procapsid where-in the ssRNA genomic precursors are packaged in an energy-dependent process (Fig. 4). Our results suggest a co-assembly strategy for the hPBV capsid proteins and the genomic precursor ssRNAs.

The hPBV is a naked virus and has no lipid structures. In contrast, phi6 has evolved to enter its host membranes by using its lipid-protein envelope. The envelope assembly of the phi6 is largely unknown. The major envelope protein P9 and the non-structural protein P12 are essential in the process (Mindich et al., 1976). This study revealed details of the phi6 envelope assembly mechanism and determined the minimum requirements for the phi6-specific vesicle formation. Interesting details of the virion envelopment process could be revealed using *E. coli* co-expression systems. It was observed that the phi6 major envelope protein is the only protein needed to produce membranous structures and phi6-specific vesicles in *E. coli*. The hypothesis that P12 might act as a protease inhibitor in the envelopment process got also support, since the P9-GFP fusion protein was protected from proteolysis only when P12 was co-expressed with P9.

This Thesis provides new information about the molecular details of dsRNA virus replication and assembly which are inherently interesting but may also have future use in biotechnological applications. RdRps that efficiently replicate heterologous template RNAs in the absence of other proteins are relatively rare. In this study a new RdRp from human PBV with these properties has been discovered and characterized enzymatically. This polymerase has potential for use in biotechnology in RNA production. Also expression of membrane proteins and vesicles has high interest in biotechnology. This Thesis introduces a prokaryotic vesicle production system which allows production of cytoplasmic membrane vesicles in the absence of host background vesicle production. The phi6-specific vesicles have potential use in biomedicine as drug carriers. We showed that heterologous protein GFP can be added to the vesicle as a P9 fusion partner. In the future, antibodies or ligands could be added onto these vesicles which would allow them to be targeted to specific cells. In this Thesis, it was also shown that addition of a heterologous protein to this vesicle production system is possible by co-expressing it with wt P9 and P12. In addition it was shown that non-structural protein P12 has potential use in membrane protein expression as proteolysis inhibitor. Thus, the knowledge of the molecular details of the dsRNA viruses does not only help to feed our curiosity about the nature but may provide interesting and diverse solutions for the biotechnological challenges in the future.

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